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Novel herpesvirus in the critically endangered Galapagos pink land iguana

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ABSTRACT

Virus surveillance in wildlife is important to understanding ecosystem health, taxonomy, and evolution. Nevertheless, viruses in reptiles, and specifically in squamates, continue to be understudied. Herein, we conducted a health assessment on the critically endangered Galapagos pink land iguana (*Conolophus marthae*) and the vulnerable Galapagos land iguana (*Conolophus subcristatus*). We collected oral and/or cloacal swabs from 47 clinically healthy iguanas and tested for adenovirus (cloacal swabs, $n = 47$) and herpesvirus (oral swabs, $n = 45$) using broad-spectrum PCRs. Two out of 38 (5.3 %) Galapagos pink land iguanas tested positive for herpesvirus, while no herpesvirus was detected in all Galapagos land iguanas ($n = 7$). Both herpesviral sequences were identical between them and divergent (61.9 % amino acid identity) when compared to the closest herpesvirus sequences available in GenBank/EMBL/DDBJ. The genetic distance between this and other herpesviruses is consistent with its classification as a novel virus species. All iguanas were negative for adenovirus. This is the first description of a herpesvirus in iguanas of the Galapagos islands, and the first report of a potential pathogen for the iconic Galapagos pink land iguana. Further research is needed to understand the implications of this virus in the conservation and management of one of the most endangered iguana species in the world.

1. Introduction

Reptile virology is a growing field of knowledge (Marschang, 2019). Among the viruses described in reptiles, herpesviruses (HVs, family *Orthoherpesviridae*) have been detected in the orders Testudine (turtles), Crocodilia (crocodilians), and Squamata (lizards and snakes) (Ariel et al., 2011; Marschang, 2011). Currently, the already identified and characterized reptilian HV species belong to the subfamily *Alphaherpesvirinae*, with the exception of Iguanid herpesvirus 2, still unclassified (Marschang, 2019; Okoh et al., 2021).

Among reptile HVs, those affecting testudines are the most studied and characterized (Marschang, 2011). Herpesviral infections in testudines are associated with skin lesions, neoplasia, oral and hepatic lesions, respiratory signs, and neurological disease, although several HVs have been described in clinically healthy animals (Marschang et al., 2001; Sim et al., 2015; Yonkers et al., 2015; Lindemann et al., 2018). Herpesvirus descriptions in squamates are sporadic, and little is known about their host specificity, pathogenicity, or classification (Marschang et al., 2021). Iguanid herpesvirus 1 was the first squamata HV, described from cell cultures of spleen, kidney, and heart of a green iguana (*Iguana iguana*) (Clark and Karzon, 1972). Subsequently, iguanid herpesvirus 2 was isolated in the San Esteban Chuckwalla (*Sauromalus varius*), associated with diffuse hepatic necrosis (Wellehan et al., 2003). A novel HV, preliminarily named iguanid herpesvirus 3, was recently described in swab samples from three green iguanas, one of them presenting a head abscess, while the other two remained clinically healthy throughout the study period (1 year) (Marschang et al., 2021). Several other HVs have been described in squamates (Marschang et al., 2021). Among them, a

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green lizard HV was identified in papillomatous lesions as an incidental finding of an otherwise subclinical infection of a green iguana (Raynaud and Adrian, 1976). Three novel HVs were identified by PCR in lizards (*Gerrhosaurus* spp.) with stomatitis and were named gerrhosaurid herpesviruses 1–3 (Wellehan et al., 2004a). Herpesvirus-like particles were detected by transmission electron microscopy in the liver of a green iguana with acute hepatocellular necrosis, with similar characteristics to those described for iguanid herpesvirus 1 (Wilkinson et al., 2005). More recently, a novel HV was identified in several smooth green snakes (*Opheodrys vernalis*) with oropharyngeal squamous cell carcinoma (Lovstad et al., 2019) and another HV was detected in a banded Gila monster (*Heloderma suspectum cinctum*) with a gingival nodule on the mandible (Goe et al., 2016).

The Galapagos Islands are located in the Eastern Tropical Pacific, almost 1000 km off the coast of mainland Ecuador (Escobar-Camacho et al., 2021). This archipelago is home to three endemic species of land iguanas: (i) the Galapagos land iguana (*Conolophus subcristatus*), located in Fernandina, Isabela, Santa Cruz, Plaza Sur, Seymour Norte (a translocated population), and Baltra (a repatriated population) islands; (ii) the Barrington land iguana (*Conolophus pallidus*), which only occurs on Santa Fe island; (iii) and the Galapagos pink land iguana (*Conolophus marthae*), restricted to the Wolf Volcano, in Isabela Island (Gentile and Snell, 2009; Arteaga et al., 2022). The Galapagos land iguana and Barrington land iguana are listed as Vulnerable, whereas the Galapagos pink land iguana is classified as Critically Endangered by the International Union for Conservation of Nature (Gentile, 2012; Gentile and Grant, 2020; Kumar et al., 2020), with the latter sustaining an estimated population of less than 300 adult individuals (Colosimo et al., 2022a). Major threats to these endemic iguanas include invasive species, climate change, a restricted distribution area (Galapagos pink land iguana and Barrington land iguana), a very small population size, and lack of recruitment (Galapagos pink land iguana) (Tapia et al., 2018; Colosimo

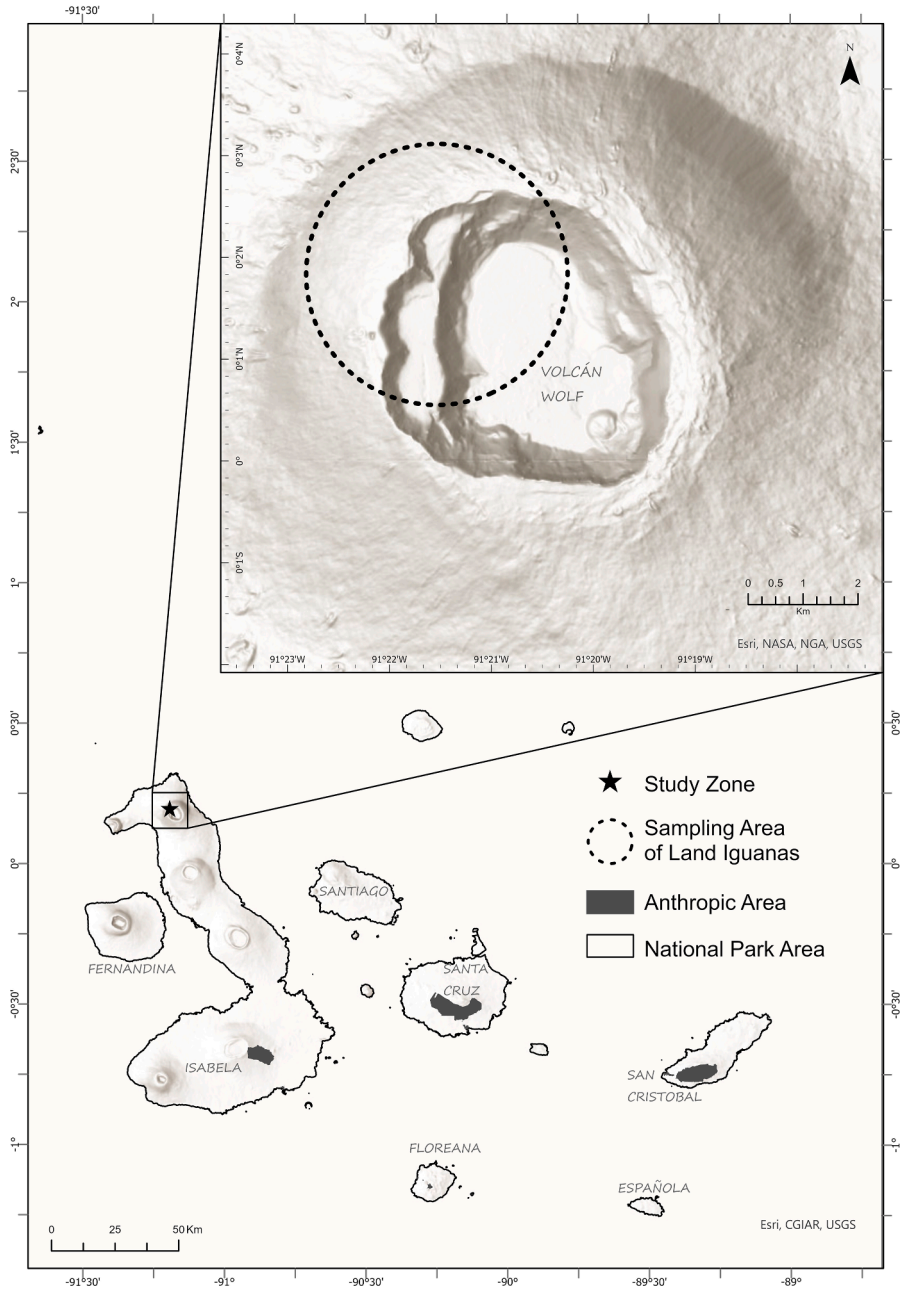

Fig. 1. Land iguana sampling area in Wolf Volcano to study infectious agents (herpesvirus and adenovirus) within the Galapagos archipelago. Source of the map: own source.

et al., 2022b).

Despite the recognition of Galapagos land iguanas as flagship species for the archipelago (Gentile et al., 2016), little is currently known about their health status (Colosimo et al., 2022a), and the presence and prevalence of infectious agents in these species. Thus, our goals were to survey for the presence of HV and adenovirus (AdV) in Galapagos pink land iguanas and Galapagos land iguanas in order to contribute to their conservation in the wild.

2. Materials and methods

2.1. Study area and sample collection

This study was conducted at the Wolf Volcano (0.03792° N; 91.36324° W), in Isabela Island, northwestern Galapagos archipelago (Fig. 1). All animals were sampled from an area comprising approximately 6 km^2, at an altitude of 1600–1700 m. A total of 47 iguanas (nine Galapagos land iguanas and 38 Galapagos pink land iguanas) were captured as part of a broader health assessment of iguanas from Wolf Volcano performed in September 2019 and April 2021 (Colosimo et al., 2022a). Following capture and immobilization, cloacal and oral swabs were collected from each individual using a sterile cotton swab (Puritan Sterile Products Company LLC, Maine) placed individually in 2 ml sterile cryovials. In two Galapagos pink land iguanas oral swabs were not collected, resulting in 45 oral swab samples (seven Galapagos land iguanas and 38 Galapagos pink land iguanas) to be tested. All samples were kept frozen at −30 °C for up to 2 months until analyses. Cloacal swab samples were analyzed for AdV ($n = 47$), while oral swab samples were tested for HV ($n = 45$). Animal capture, handling, and sampling were performed according to a protocol approved by the Galapagos National Park. Field activities and sample collection were carried out under the Galapagos National Park annual research permits PC-92–19 and PC-04–21 and addenda.

2.2. Molecular analyses

Each swab sample was incubated for 1 hour at room temperature with 100 μl of sterile phosphate buffered saline (PBS). Subsequently, total DNA was extracted using the DNeasy Blood & Tissue extraction Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. Cloacal swabs were tested for AdV using a broad-spectrum nested PCR method with consensus primers targeting a 330 bp region of AdV's DNA-dependent DNA polymerase gene (Wellehan et al., 2004b). Oral swab samples were tested for HV using a broad-spectrum nested PCR assay, which amplifies a fragment of approximately 215 - 315 bp of the DNA polymerase gene (VanDevanter et al., 1996). PCR products were visualized in 1.5 % agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA). To improve the quality of the amplicons, we retested the preliminary HV-positive samples with a modified VanDevanter et al. (1996) protocol. Briefly, we added 2.5 μl of GC Enhancer to both external and internal PCR reactions and increased the sample volume amount up to 4 μl per reaction. Thermal cycles were also adjusted as follows: 95 °C 10 min; 40X (94 °C 30″, 46 °C 60″, 72 °C 60″); 72 °C 7 min.

After purification with ExoSAP-IT (Affymetrix USB products, Santa Clara, California, USA), amplicons of the expected size were directly sequenced in both directions. We considered a sample positive if a clear sequence was obtained. The obtained forward and reverse sequences were aligned with Mega 7.0 to construct the consensus sequence, and the primers were edited out (Kumar et al., 2016). Nucleotide (nt) and deduced amino acid (aa) consensus sequences were subjected to BLAST searches. Subsequently, the percentage of identity of the obtained nt and aa sequences with the closest ones from GenBank/EMBL/DDBJ was calculated based on p-distance ([1 − *p distance*]*100). We also compared the obtained sequences to those herpesviral sequences previously described in tortoises from the Galapagos archipelago, including sympatric tortoises of Wolf Volcano (Nieto-Claudin et al., 2021). Nucleotide and aa phylogenetic trees were constructed on Mega 7.0 using a maximum likelihood algorithm with a bootstrap frequency of 1000 replications. The best evolutionary model for nt and aa phylograms was selected using JModelTest 2 (Fig. 2) and ProtTest 3 (Fig.3), respectively (Darriba et al., 2011; 2012).

3. Results

All sampled iguanas from both species were considered clinically healthy based upon physical examination, scaled mass index, and biochemical and hematological parameters (Colosimo et al., 2022b). None of the iguanas tested positive for AdV. Overall, two out of the 45 tested iguanas were HV-positive (4.4 %; 95 % CI 0.0–9.8). Both individuals were Galapagos pink land iguanas (2/38; 5.3 %; 95 % CI 0.0–11.7): a female (Case 811) and a male (Case 922) adults sampled in 2019 and 2021, respectively. We obtained two herpesviral sequences of 164 (#811) and 192 bp (#922) in length, which were submitted to GenBank under accession numbers OP661354 and OP661355, respectively. The aligned portion of the two sequences was identical. We selected the longest Galapagos pink land iguana HV sequence to calculate the nt and aa similarities to the closest one from GenBank. The 192 bp sequence presented the highest nt identities (75.6 % and 77.9 %) to a murine HV (KF155695) found in a house mouse (*Mus musculus*) of the Republic of the Congo and to a bat HV retrieved from a greater short-nosed fruit bat (*Cynopterus sphinx*) of China (KR261896) (Fig. 2). When aligned, KF155695 and KR261896 sequences were shorter than the sequence type obtained in our study, overlapping in only 68 and 78 bp, respectively. The highest aa identity (61.9 %) was to the iguanid herpesvirus 2 described in a San Esteban Chuckwalla (GenBank accession n°. YP009664708) (Fig. 3). The HV nt sequence from the San Esteban Chuckwalla has 100 % coverage and the same length as the one from the pink land iguana #922, with 63.5% nt similarity.

When we compared the Galapagos pink land iguana HV to (i) the HVs found in Galapagos giant tortoises, specifically to the Chelonoidis herpesvirus 1 (CheHV1) described in several tortoise species, including Wolf Volcano giant tortoises (*Chelonoidis becki*), and (ii) to the CheHV2, reported only in Wolf Volcano giant tortoises, our sequence presented nt identities of 53.1 %, 53.7 %, and 54.4 % to CheHV1 (GenBank accession n°. OU508388, OU508389, and OU508390, respectively) and 49.4 % to CheHV2 (OU508391). The aa identity between the novel HV sequence found in Galapagos pink land iguanas and CheHV1 and CheHV2 was 47.2 % and 41.4 %, respectively. In the phylogenetic tree, our HV sequence did not cluster with other squamate HVs with a bootstrap support equal to or higher than 70 % (Fig. 2).

4. Discussion

To the authors' knowledge, this is the first HV description in a Galapagos iguana species and the first report of a potential viral pathogen in the critically endangered Galapagos pink land iguana. The virus was retrieved from two clinically healthy pink land iguana adults. In squamates, HVs have been identified in clinically healthy individuals (Marschang et al., 2021), but have also been associated with stomatitis, enteritis, hepatitis, papillomas, and carcinomas (Wellehan et al., 2004a; Hughes-Hanks et al., 2010; Goe et al., 2016; Lovstad et al., 2019). In most cases, severe disease due to HVs is only observed in young or immunosuppressed animals, or following infection of an incidental host (Marschang, 2019). Nevertheless, HV-infected individuals that survive initial infection can be considered lifelong carriers (Marschang, 2019). Of note, apparently healthy reptiles can shed HVs, as observed in an apparently healthy captive green iguana that reportedly shed iguanid herpesvirus 3 for a year (Marschang et al., 2021).

Environmental factors, particularly temperature, are known to influence the immune system of reptiles, although additional factors such as pollutants or concomitant infections may also play an important role in the outcome of viral infections (Marschang, 2011). These factors can

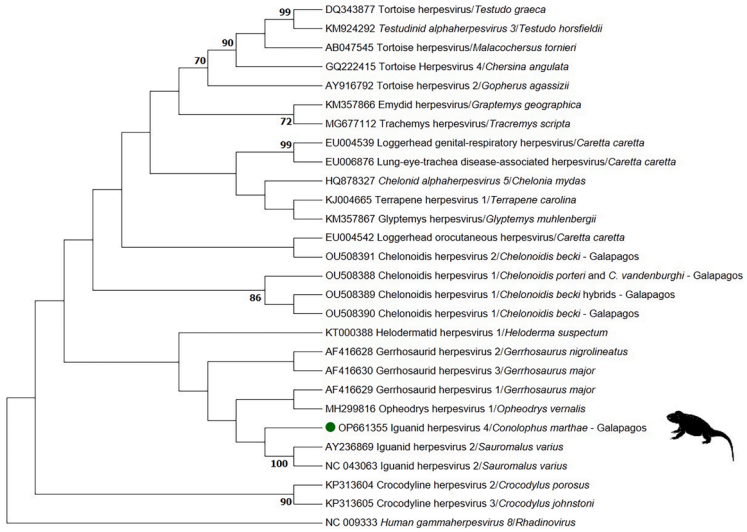

Fig. 2. Maximum likelihood phylogram of the nucleotide alignments of the consensus herpesvirus sequences obtained in Galapagos pink land iguana (*Conolophus marthae*), and in other reptiles of the orders Squamata, Testudines, and Crocodilia. *Human gammaherpesvirus 8* was selected as outgroup. The selected model for the nucleotide was General Time Reversible model. The reliability of the phylogram was tested by 1000 replicate bootstrap analyses omitting values below 70.

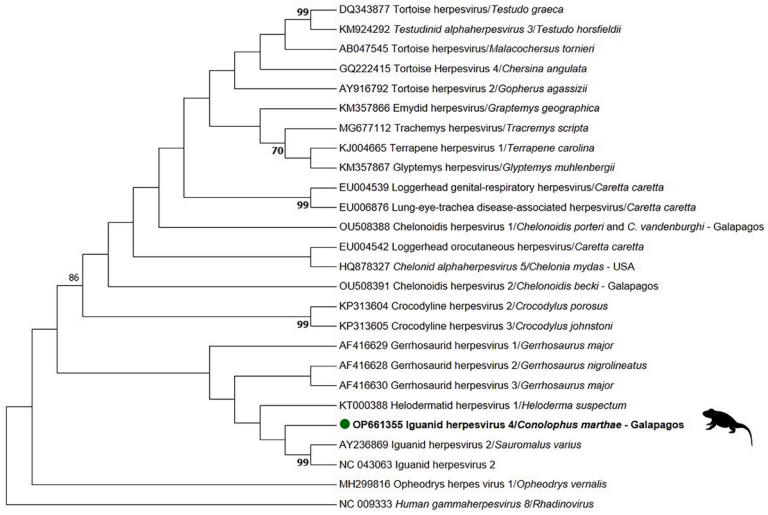

Fig. 3. Maximum likelihood phylogram of the deduced amino acid alignments of the consensus herpesvirus sequences obtained in Galapagos pink land iguana (*Conolophus marthae*), and in other reptiles of the orders Squamata, Testudines, and Crocodilia. *Human gammaherpesvirus 8* was selected as outgroup. The selected model for the amino acid phylogram was Le Gascuel. The reliability of the phylogram was tested by 1000 replicate bootstrap analyses omitting values below 70.

cause immunosuppression, the subsequent reactivation of latent HVs and lead to clinical disease (Woźniakowski and Samorek-Salamonowicz, 2015). Of note, despite this novel HV was detected in apparently healthy pink land iguanas, its pathogenicity is unknown. In Galapagos, the critically endangered pink land iguana has recently undergone new conservation strategies led by the Galapagos National Park and other institutions, including a potential captive-breeding program and/or translocation or reintroduction program (Colosimo et al., 2022b). Wildlife manipulation, transportation, captivity, and overcrowding are stressful factors that may promote immunosuppression (Parker and Deem, 2012). Therefore, screening of microbial agents in Galapagos pink land iguanas - including this novel HV - is imperative to prevent unnecessarily and avoidable morbidity and mortality, as well as to guarantee the success of ongoing conservation programs.

Despite the short size of the obtained sequence type, the high genetic distance seen between our HV and the closest HVs available at GenBank/DDBJ/EMBL, and its detection in a novel host species, is consistent with the placement of this virus as a novel species, tentatively named iguanid alphaherpesvirus 4 (IgHV4). Nevertheless, the scientific name should be provided by the ICTV. The IgHV4 was also divergent when compared to two novel HVs described in 2021 in Wolf Volcano giant tortoises (*Chelonoidis becki*) (Nieto-Claudin et al., 2021), an endemic giant tortoise species whose habitat is restricted to Wolf Volcano, and that overlaps with both the Galapagos land iguana and the pink land iguana (Caccone et al., 2017). These differences suggest that Galapagos pink land iguanas are likely the natural host of IgHV4. Although sympatric in Wolf volcano, tortoises and iguanas do not share the same hemoparasites (Fulvo, 2010). In fact, a recent genetic analysis of Apicomplexa hemogregarines present in sympatric tortoises and iguanas in Wolf volcano indicated strong divergence and host specificity (Fulvo, 2010). Such data further support the existence of coevolutionary relationships between hosts and parasites/viruses in Galapagos reptiles, as described in other squamates of the Bahamas archipelago (Colosimo et al., 2021).

Adenovirus was not detected in the present study; however, it has been described in several squamate species (Ball et al., 2014; Prado-Irwin et al., 2018), associated with morbidity and mortality in

captive animals (Moormann et al., 2009; Hyndman and Shilton, 2011; Ascher et al., 2013). In reptiles of the Galapagos archipelago, four nucleotide AdV strains were described in apparently health free-living and captive giant tortoises from Santa Cruz Island and Alcedo Volcano, while no AdV positive tortoises were detected in Wolf Volcano (Nieto-Claudin et al., 2021), where our study was conducted. To this date, the presence of AdV in Galapagos squamates is unknown.

Diversification within the Galapagos iguana clade started millions of years ago (Rassmann, 1997; Gentile and Snell, 2009; MacLeod et al., 2015), most likely following a single colonization event from the mainland. Extending the viral surveillance to continental reptiles would provide crucial information on whether the coevolutionary relationship originated prior or after the colonization of the island. Further investigations, following the identification of three different reptile HV sequences in Wolf Volcano, will help clarify if this isolated environment could act as an adequate "field laboratory" to study reptilian HV evolution and virus-host coevolution (Kaján et al., 2020).

5. Conclusion

This is the first HV description in Galapagos iguana species. Further research is necessary to obtain the complete genome of this novel HV, understand whether this virus may be pathogenic to the endangered pink land iguanas or potentially to other Galapagos land or even marine iguanas, and to support the conservation of one of most threatened reptile species in the world.

CRediT authorship contribution statement

Ainoa Nieto-Claudín: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Carlos Sacristán:** Data curation, Software, Writing – original draft, Writing – review & editing. **Sharon L. Deem:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **Gregory A. Lewbart:** Investigation, Writing – review & editing. **Giuliano Colosimo:** Investigation, Writing – review & editing. **Fernando Esperón:** Funding acquisition, Methodology, Writing – review & editing. **Christian Sevilla:** Investigation, Writing – review & editing. **Gabriele Gentile:** Funding acquisition, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analyzed during this research are included in the manuscript. The nucleotide sequences obtained in this research were submitted to GenBank under accession numbers OP661354 and OP661355.

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Ethics approval

The study was conducted in accordance with the Galapagos National Park Directorate. Animal capture, handling, and sampling were performed according to a protocol approved by the Galápagos National Park. Field activities and sample collection were carried out under the Galápagos National Park annual research permits PC-92–19 and PC-04–21 and addenda, granted to GG. The protocol was approved by the Institutional Review Board of Galapagos National Park Directorate. This research complies with institutional ethical guidelines of the Galapagos National Park Directorate, under annual research permits authorized by the environmental agency.

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Declaration of Generative AI and AI-assisted technologies in the writing process

Not applicable.

Permits and ethical statement

Field activities and sample collection were carried out under the Galápagos National Park Directorate (GNPD) annual research permits PC-92–19 and PC-04–21 and addenda, granted to Gabriele Gentile, which authorized all the activities performed. The study followed all the ethics and animal handling protocols of the GNPD and was authorized by that institution.

Consent for publication

Not Applicable.

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